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Mitochondrial DNA-based phylogeography of the large ringlet *Erebia euryale* (Esper, 1805) suggests recurrent Alpine-Carpathian disjunctions during Pleistocene (Nymphalidae, Satyrinae)

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Abstract. Most species of the butterfly genus *Erebia* are high altitude specialists, in which territorial fragmentation is associated with distinct genetic patterns. This is also true for the large ringlet, *Erebia euryale* (Esper, 1805), a species widespread across European mountain systems. Previous molecular studies revealed four lineages: two in the Alps, coinciding with the ssp. *adyte* and *isarica*, one in the Pyrenees and Cantabria (ssp. *pyraenaeicola*), and one in the Carpathians and the Balkans (ssp. *syrmia*). Two morphological subspecies inhabiting delimited ranges in the southern Alps (ssp. *pseudoadyte* and *kunzi*) were not included in these studies. To further our understanding of the relationships between populations, both the Alpine and the extra Alpine ones, we sequenced 1,496 bp of the COI gene in 16 Alpine and Jurassian populations and analysed them in combination with published Pyrenean and Carpathian sequences. The resulting haplotype network shows five lineages, congruent with the morphologic delineation of subspecies. Based on the current distribution ranges and genetic affinities, we reconstructed a pre-Würm phylogeographic scenario. This suggests an initial split resulting in an Alpine and a Carpathian clade, probably of Carpathian origin. Within the Alps, three subspecies subsequently differentiated, probably during several glacial cycles, generating ssp. *adyte*, *pseudoadyte* and *kunzi*. In parallel, the Carpathian clade underwent a second Alpine–Carpathian disjunction and differentiated into ssp. *euryale* and *syrmia* in the Carpathians, and ssp. *ocellaris* and *isarica* in the eastern Alps, revealing a heterogeneous origin of the *E. euryale* subspecies across the Alps. The Pyrenean and Jurassian populations are a relatively young divergence in the western part of the species' range.

Introduction

High altitude organisms in general have the same insular distribution patterns as their habitats (a "continental–insular fauna": Varga 1996). In their fragmented territory gene flow is effectively interrupted, resulting in a geographical pattern of genetic variation (Varga 1996; Varga and Schmitt 2008). This phylogeographic structure was mainly shaped during the Quaternary climate oscillations (Hewitt 1996; Comes and Kadereit 1998; Schmitt 2007). The succession of warm and cold periods caused repeated altitudinal range shifts of mountain biota. The classic idea that, during glacial periods, cold-adapted species were widespread in the plains (Reinig 1938; Holdhaus 1954;

de Lattin 1967; Muster and Berendonk 2006) has been superseded (Schmitt et al. 2006; Schmitt 2007). The glaciated Alps were flanked by permafrost tundra in the north, and in the south by dry cold-steppe (van Andel and Tzedakis 1996; Tzedakis et al. 2002; Monegato et al. 2007). Both were arid and treeless (Lang 1994; Müller et al. 2003; Stojakowits et al. 2020), thus hostile to alpine species dependent on moisture and wind shelter. For these organisms, the plains were liveable only during a relatively short period in the transition to a warm climate. This enabled them to colonise distant, nowadays ecologically isolated mountain ranges (de Lattin 1967; Hewitt 1999). Under pleniglacial conditions, permanently liveable circumstances were restricted to discrete, climatologically favoured areas along the alpine borders (Stehlik 2000; Schönswetter et al. 2005; Schmitt 2009). For an increasing number of plant species these peripheral refugia have been identified through a fine scale analysis of their phylogeographic patterns (Stehlik et al. 2002; Schönswetter et al. 2003; Tribsch and Schönswetter 2003; Schönswetter et al. 2004). To the best of our knowledge, no such analysis exists of Alpine butterfly species. Nunatak survival, as supposed for some alpine plants (Stehlik 2000, 2003; Parisod and Besnard 2007), has not been evidenced in butterflies yet.

The Holarctic ringlet butterfly genus *Erebia* is amongst the most intensively studied alpine insect groups, due to its high species and subspecies richness across montane/alpine and boreal/arctic biomes. The genus most likely originated in Asia, and colonised Europe some 17–23 million years (My) ago (Peña et al. 2015). It underwent a broad diversification into some dozens of species during Miocene and Pliocene (Martin et al. 2000; Peña et al. 2015; Wiemers et al. 2020), followed by a fast radiation caused by the Pleistocene glacial cycles. These most recent differentiations as a rule were intraspecific (Vila et al. 2005; Schmitt et al. 2006; Schmitt et al. 2014; Hinojosa et al. 2018; Cupedo and Doorenweerd 2020), and in a few cases on species level (Martin et al. 2002; Albre et al. 2008; Wiemers et al. 2020). The large ringlet, *Erebia euryale* (Esper, 1805) is one of the most widespread and most variable species of the genus, with sixteen morphological subspecies described (Cupedo 2010). Five of these are endemic to the Alps: *adyte*, *pseudoadyte*, *kunzi*, *isarica* and *ocellaris*. Their territories are separated by extremely narrow intergradation zones (Rezbanyai-Reser 1991; Sonderegger 2005; Cupedo 2014), as is common in closely related *Erebia* taxa (Schmitt and Müller 2007; Lucek et al. 2020). In contrast, the subspecies *isarica* and *ocellaris* are separated by a hybrid zone tens of kilometres wide (Cupedo 2010). Two molecular studies addressed the genetic relationships of the central European taxa. Schmitt and Haubrich (2008) identified two allozyme based lineages in the Alps, coinciding with the morphologic ssp. *adyte* and *isarica*. The ssp. *ocellaris* and *isarica* were found genetically identical, the ssp. *pseudoadyte* and *kunzi* (both described two years later) were not sampled. Outside the Alps, their study identified a lineage in the Pyrenees (concordant with ssp. *pyraenaeicola*), and one in the Southern Carpathians and the Balkans (ssp. *syrmia*). A mtDNA based study (Vila et al. 2011) revealed that (i) the populations from the Pyrenees and Cantabria represent a single lineage, and (ii) the *ocellaris* and *isarica* samples are phylogenetically closer to *syrmia* than to each other. Paučulová et al. (2017) found two genetic lineages in the Carpathians and the Sudeten Mts, coinciding with the morphologic ssp. *euryale* and *syrmia*, and established the border between them. Ssp. *euryale* inhabits the Sudeten Mts (Jeseník, Krkonoše) and the Western Carpathians (Tatra, Fatra), whereas ssp. *syrmia* inhabits the Eastern and Southern Carpathians.

To date, the subspecies *pseudoadyte* and *kunzi* are only morphologically defined. Although very similar to *adyte* and *ocellaris* in their wing pattern, they exhibit consistent differences in the male genital characters. Ssp. *kunzi* occurs in two forms: western and eastern *kunzi* (Fig. 1B). Both geographically and wing-morphologically the western form is intermediate between *pseudoadyte*

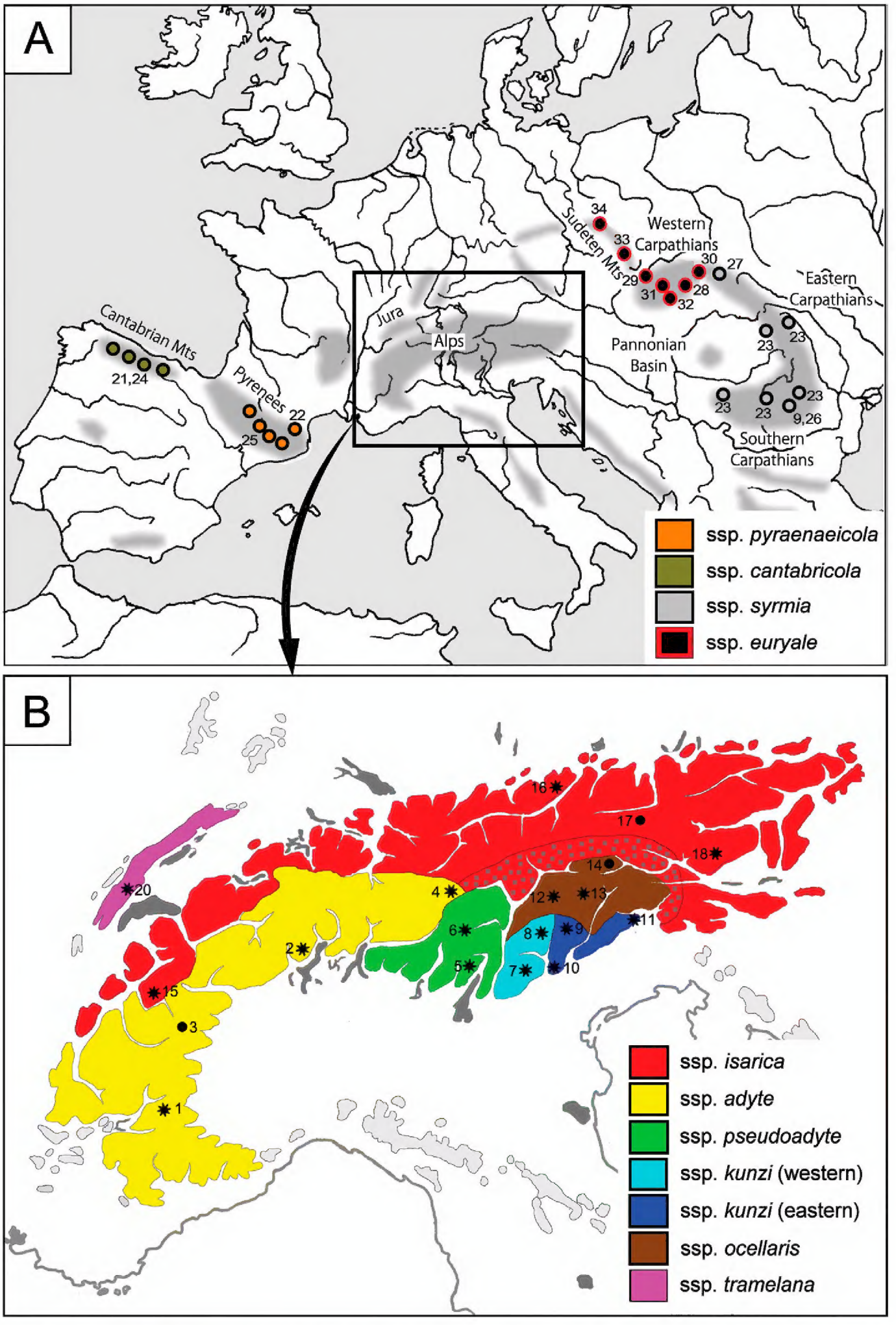

Figure 1. Overview of the locations listed in Table 1. **A.** Locations outside the Alps and the Jura, sequences retrieved from GenBank. Black rectangle: area covered by Fig. 1B; **B.** Locations in the Alps and the Jura. **Stars** – sampling locations, **dots** – sequences mined from GenBank**, coloured areas** – distribution of morphologic subspecies after Willien (1985), Sonderegger (2005), Cupedo (2010) and personal observations, **dotted area** – transitional populations *isarica*/*ocellaris*, **dark grey** – lakes and coastline. Sample numbers refer to Table 1.

and eastern *kunzi*. Because of genital characters it was nonetheless regarded as *kunzi*, rather than of hybrid origin (Cupedo 2010). At the very few known contact sites, both between *adyte* and *pseudoadyte* and between *kunzi* and *ocellaris*, morphological intergradation zones are extremely narrow, suggesting reduced reproductive compatibility (Cupedo 2014).

The number of genetic lineages of *E. euryale* in the Alps thus may vary between two and five, depending on the underlying data. In order to obtain one dataset covering all recognized and potential lineages, we extended previously published mtDNA data (Dincă et al. 2010; Vila et al. 2011; Paučulová et al. 2017) with 15 Alpine populations and a Jurassian one. We sequenced both the 5'P section of the Cytochrome C Oxidase I gene (the "barcode section"), and the 3'P part, which has proven to be more informative in some *Erebia* species (Vila et al. 2005; Vila et al. 2011), although not all of them (Cupedo and Doorenweerd 2020). The aim of our study is to identify the mtDNA based genetic lineages of *E. euryale* in the Alps, to establish their relation to morphologic subspecies, to clarify their phylogenetic relations and their connections with the Jura, Pyrenees (including Cantabria), the Carpathians and the Sudetes, and to reconstruct their phylogeographic history. With these objectives, we addressed six specific questions: (1) Are the subspecies *pseudoadyte* and *kunzi* distinct lineages, different from their look-alikes *adyte* and *ocellaris*, respectively? (2) If so, are the morphologically intermediate populations ('western *kunzi*') rightly assigned to ssp. *kunzi*? (3) Do the ssp. *isarica* and *ocellaris* represent one or two genetic lineages? (4) What is the genetic relation between the Alpine lineages and those from the Jura, the Pyrenees, the Sudetes and the Carpathians/Balkans? Having clarified these issues, we (5) performed a fine-scale reconstruction of the Würm glacial refugia of the Alpine lineages, as a setup for (6) a reconstruction their pre-Würm phylogeography, in conjunction with the Jurassian, Pyrenean and Carpathian lineages.

Material and methods

Study species

Erebia euryale finds its ecologic optimum in edges and clearings of the timberline forest, irrespective of the forest association (conifer or beech forest), or the underlying bedrock (calcareous or siliceous). Above the timberline, it can be abundant in the green alder shrubs (*Alnetum viridis*) and in the alpenrose heath vegetation (*Rhododendro–Vaccinietum*). Locally, it descends into the montane zone or ascends into the alpine meadows (Gonseth 1987; Sonderegger 2005). The larvae feed on grasses, their development is biennial (Sonderegger 2005; Klecková et al. 2015).

Sampling

Adult individuals were collected at 16 localities (Fig. 1 and Table 1). As an outgroup, two males were sampled of *E. ligea* (Linnaeus, 1758), the nearest relative of *E. euryale* (Dincă et al. 2010; Peña et al. 2015; Dincă et al. 2021). Netted individuals were paralysed with ethyl acetate vapour, and two legs were transferred to ethanol 96% before death. Specimens included in this study are all registered on the Barcode of Life Database (Ratnasingham and Hebert 2007) with detailed information on collecting localities and identification (dataset https://doi.org/10.5883/DS-EURYALE) Vouchers are deposited in the collection of Naturalis Biodiversity Center (RMNH; Leiden, the Netherlands). Additional COI sequences were retrieved from BOLD and GenBank, from localities not represented in our samples. These covered bp 1–658 (Dincă et al. 2010), bp 668–1483 (Vila et al. 2011) or bp 305–1475 (Paučulová et al. 2017) (Table 1, Suppl. material 1: Table S1).

Table 1. Sampling data of *Erebia euryale* and *Erebia ligea* (outgroup) underlying the haplotype networks in Fig. 2. **No** – sample number, **Voucher ID** – of own samples (prefix RMNH.INS.), **N** – sample size, **3'P, 5'P, 305-1475** – COI segment sequenced. Samples marked with asterisk are mined from GenBank.

No	Subspecies	Author	Massif	Locality	Ctry	Coordinates (DDM)	Alt (m)	Date	Voucher ID	N	3'P	5'P	305-1475
1	*adyte*	(Hübner, 1822)	Cottian Alps	Risoul	FR	44°38.07'N, 006°37.95'E	5525	13/07/2012	552442–552452	11	x	x	
2	*adyte*	(Hübner, 1822)	Penninic Alps	Cheggio	IT	46°05.84'N, 008°06.06'E	1760	09/07/2012	559813–559822	8	x	x	
*3	*adyte*	(Hübner, 1822)	Graian Alps	Val d'Isère	FR	45°27'N, 006°59'E	2000–2100	04/08/2003		10	x		
4	*adyte*	(Hübner, 1822)	W. Rhaetian Alps	Langtauferertal	IT	46°49.49'N, 010°40.82'E	2000	29/07/2009	544671–544685	6	x	x	
5	*pseudoadyte*	Cupedo, 2010	Garda Pre-Alps	Passo Tremalzo	IT	45°50.78'N, 010°40.92'E	1490	27/07/2013	559888–559897	10	x	x	
6	*pseudoadyte*	Cupedo, 2010	Adamello	Val Genova	IT	46°10.81'N, 010°38.06'E	1390	28/07/2013	556626–556637	11	x	x	
7	*kunzi*	Heinkele, 2007	Dolomites-Latemar	Gallio	IT	46°55.58'N, 011°33.62'E	1620	21/07/2013	559865–559876	12	x	x	
8	*kunzi*	Heinkele, 2007	Dolomites-Latemar	Passo Brocon	IT	46°07.24'N, 011°41.43'E	1620	20/07/2013	559853–559864	12	x	x	
9	*kunzi*	Heinkele, 2007	Dolomites-Feltre chain	Passo Palughet	IT	46°10.97'N, 011°54.90'E	1850	03/08/2008	544662–544670	4	x	x	
10	*kunzi*	Heinkele, 2007	Venetian Pre-Alps	Monte Grappa	IT	45°52.97'N, 011°47.69'E	1630	22/07/2013	559877–559887	11	x	x	
11	*kunzi*	Heinkele, 2007	Venetian Pre-Alps	Monte Cavallo	IT	46°07.95'N, 012°31.36'E	1350	17/07/2013	556602–556613	11	x	x	
12	*ocellaris*	Staudinger, 1861	Dolomites	Karer Pass	IT	46°25.12'N, 011°36.04'E	1780	31/07/2013	556614–556625	12	x	x	
13	*ocellaris*	Staudinger, 1861	Dolomites	Passo Pordoi	IT	46°28.92'N, 011°47.30'E	1940	01/08/2013	559898–559907	10	x	x	
*14	*ocellaris*	Staudinger, 1861	Dolomites	Kalkstein	AT	46°48'N, 012°19'E	1600	15/08/2005		5	x		
15	*isarica*	Heyne, 1895	Graian Alps	Cormet de Roselend	FR	45°41.62'N, 006°39.49'E	1760	25/07/2012	559823–559828	5	x	x	
16	*isarica*	Heyne, 1895	N Tyrol limestone Alps	Rofan Mountains	AT	47°27.82'N, 011°49.56'E	1750	05/08/2013	559829–559840	12	x	x	
*17	*isarica*	Heyne, 1895	Nieder Tauern	Obertauern	AT	47°15'N, 013°34'E	1800–2000	18/08/2005		6	x		
18	*isarica*	Heyne, 1895	Gurktal Alps	Turracher Höhe	AT	46°55.53'N, 013°53.05'E	1890	16/07/2013	559841–559852	12	x	x	
*19	*syrmia*	Fruhstorfer, 1909	S. Carpathians	Bucegi Mts	RO	45°21'N, 025°31'E	1400–1600	22/07/2004		10	x		
20	*tramelana*	Reverdin, 1818	Jura	Mijoux	FR	46°21.59'N, 006°01.03'E	1520	26/07/2012	556638–556648	11	x	x	
*21	*cantabricola*	Verity, 1927	Cantabrian Mts	Cantabria	SP	42°N, 004°E–006°E		07/2006		36	x		
*22	*pyrenaeicola*	v.d. Goltz, 1930	Pyrenees	La Glèbe	FR	42°40'N, 002°13'E	1500-2000	28/07/2003		10	x		
*23	*syrmia*	Fruhstorfer, 1909	S. Carpathians	Div. loc.	RO					17		x	
*24	*cantabricola*	Verity, 1927	Cantabria	Div. loc.	SP					6		x	
*25	*pyrenaeicola*	v.d. Goltz, 1930	Pyrenees	Div. loc.	FR, AND					10		x	
*26	*syrmia*	v.d. Goltz, 1930	S. Carpathians	Bucegi Mts	RO	45°24.37'N, 025°29.86'E		2012		5			x
*27	*syrmia*	v.d. Goltz, 1930	E. Carpathians	Bukovské Mts	SK	49°05.30'N, 022°34.05'E		2011		4			x
*28	*euryale*	(Esper, 1805)	W. Carpathians	Volovské Mts	SK	48°46.95'N, 020°59.32'E		2011		5			x
*29	*euryale*	(Esper, 1805)	W. Carpathians	Great Fatra	SK	48°54.13'N, 019°04.75'E		2011		2			x
*30	*euryale*	(Esper, 1805)	W. Carpathians	Čergov Mts	SK	49°13.83'N, 021°00.45'E		2011		4			x
*31	*euryale*	(Esper, 1805)	W. Carpathians	High Tatra	SK	49°13.77'N, 020°13.20'E		2013		4			x
*32	*euryale*	(Esper, 1805)	W. Carpathians	Slovak Paradise	SK	48°53.38'N, 020°20.65'E		2013		5			x
*33	*euryale*	(Esper, 1805)	Sudeten Mts	Hrubý Jeseník	CZ	50°08.00'N, 017°23.00'E		2009		3			x
*34	*euryale*	(Esper, 1805)	Sudeten Mts	Krkonoše Mts	CZ	50°44.42'N, 015°44.42'E		2013		4			x
	Outgroup												
	E. ligea	(Linnaeus, 1758)	Garda Pre-Alps	Valle di Concei		45°55.68'N, 010°45.97'E	1590	26/07/2013	556599–556600	2			

DNA extraction, PCR and sequencing

Genomic DNA was extracted from the legs with a Macherey–Nagel NucleoMag 96 Tissue magnetic bead kit on a Thermo Fisher KingFisher flex system. Polymerase chain reaction (PCR) was used to amplify a fragment of 1,496 base pairs (bp) of the Cytochrome C Oxidase subunit I gene (COI) from the mitochondrial genome, in two parts. The first part of 658 bp, near the 5'P side of the gene, also known as the DNA barcode region (Hebert et al. 2003) was amplified using a 1:1 primer mix of LepF1 (5' ATT CAA CCA ATC ATA AAG ATA TTG G 3') and LCO1490 (5' GGT CAA CAA ATC ATA AAG ATA TTG G 3') as forward primers, and a 1:1 primer mix of LepR1 (5' TAA ACT TCT GGA TGT CCA AAA AAT CA 3') and HCO2198 (5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3') as reverse primers (Folmer et al. 1994; Doorenweerd et al. 2014). The second part that we amplified, from here on indicated as the 3'P fragment, has a 32 bp overlap with the 5'P fragment, and a target length of 870 bp. This fragment was amplified with forward primer SeqInt-COIf (5'CWT CWT TTT TTG AYC CAG CWG GAG 3') and reverse primer LepLEUr (5' CCA TTA CWT ATA RTC TGC CAT ATT 3') (Vila and Björklund 2004). M13 forward (5' TGT AAA ACG ACG GCC AGT 3') or reverse (5' CAG GAA ACA GCT ATG AC 3') tails were attached to the forward and reverse primers respectively, and used for sequencing. Each PCR reaction included 40 cycles, consisting of 3 minutes initial denaturation at 94 °C, 15 seconds cycle denaturation at 94 °C, 30 seconds cycle annealing at 50 °C, and 40 seconds cycle extension at 72 °C. A final extension at 72 °C for 5 minutes completed the reactions. Bidirectional Sanger sequencing was outsourced to BaseClear, Leiden, The Netherlands. The resulting chromatograms were checked for quality and congruence in Geneious R6.1.8 and the resulting sequences were managed using VoSeq 1.7.4 (Peña and Malm 2012) prior to uploading to BOLD (www.barcodingoflife.org) and Genbank (www.ncbi.nlm.gov/Genbank) (accessions MT762427–MT762602).

Analyses

Specimens with missing data were removed, leaving 158 *E. euryale* samples for haplotype analyses. Median-joining haplotype networks were constructed using NETWORK v10.2.0.0 (www.fluxus-engineering.com), based on different sequences: (i) Our main dataset of 1495 bp, i.e., the combined 3'P and 5'P fragments. (ii) Two subsets, of the 3'P and 5'P fragments separately, both expanded with sequences mined from GenBank. (iii) A set of sequences covering bp 305–1475, retrieved from GenBank. Because the mined 3'P sequences were 816 bp long instead of 838, we truncated our 3'P sequences to bp 816 bp. The excised nucleotides contained no mutations. Because the 5'P fragment in ssp. *syrmia* was invariable (N=17) and obviously did not contribute to the variation, we concatenated the mined 3'P and 5'P sequences of this subspecies and included them into our 3'P+5'P dataset, which we accordingly truncated to 1,474 bp.

From the main dataset we inferred a maximum-likelihood tree using IQ-TREE v1.6.10 (Nguyen et al. 2015) with 5,000 approximate likelihood ratio test repetitions (flag -alrt) and 5,000 rapid bootstraps (flag -bb). The IQ-Tree integrated ModelFinder selected TN+I+F as the best fit model according to the Bayesian information criterion, but all models scored very similar. For Bayesian tree inference and molecular dating we used RevBayes v1.0.12 (Höhna et al. 2016). We calibrated the root of the tree, the split between *E. euryale* and *E. ligea*, at 3.49 My to match the estimate that was based on a four gene *Erebia* wide phylogeny (Peña et al. 2015). A study that used a supertree approach with largely the same genetic data for *Erebia* but different calibration points, estimated the split between *E. ligea* and

E. euryale very similarly, at 3.58 My (Wiemers et al. 2020). We ran two independent MCMC chains in parallel for 20,000 generations, both with the GTR+G substitution model, and used Tracer v1.7.1 (Rambaut et al. 2018) to verify that this resulted in sufficiently large estimated sampling sizes (>>200).

Potential Würm glacial refugia

Reconstructing phylogeography requires insight in past area shifts. Because Würm glacial refugia form the bridge between present and pre-Würm distribution patterns, we primarily examined how the present distributions line up with potential Würm refugia. Six regions are broadly recognised as potential Würm glacial refugia: (i) In the westernmost (French pre-Alps, south of the Isère glacier) and in (ii) in the easternmost parts of the Alps (south of the Enns glacier), extended areas of unglaciated lower mountains acted as large scale refugia to alpine organisms (Schönswetter et al. 2003; Buoncristiani and Campy 2004; van Husen 2004; Schönswetter et al. 2005). (iii) Along the southern border of the Alpine arch, habitable areas were present on the south exposed slopes. These were unglaciated (Penck and Brückner 1909; Geologische Bundesanstalt (AT) 2013), and foothill topography ensured wind shelter, increased insolation, and moist conditions by orogenic precipitation and summery melt water supply, resulting in tempered micro habitats (Avigliano et al. 2002; Kropf et al. 2002; Ravazzi et al. 2004; Monegato et al. 2007). A key feature of this habitable belt is that it was heavily compartmentalized by north–south running glacial valleys (Penck and Brückner 1909; Castiglioni 2004). These valleys acted as habitat interruptions, impeding lateral dispersal. (iv) Along the northern Alpine border, a lobed ice front composed of piedmont glaciers in lateral contact, shovelled far into the foreland (Stojakowits et al. 2020). Some persisting incisions in this ice front (Keller and Krayss 2010; Reitner 2011; Heß 2013; Graf et al. 2015) offered suitable refugial conditions to sub alpine organisms (Schönswetter et al. 2005). (v) Adjacent to the eastern Alps, the Pannonian Basin has been evidenced to have had taiga like forests and even patches of temperate deciduous woodland during the Würm glacial (Rudner and Sümegi 2001; Willis and van Andel 2004; Feurdan 2005; Janovska and Pokorný 2008; Kuneš et al. 2008; Juřičková et al. 2014). (vi) The southern Pyrenean foreland offered favourable conditions even during pleniglacial periods (González-Sampériz et al. 2005). This goes to a lesser degree for the western and eastern edges of the Pyrenees (Calvet 2004). The northern Pyrenees were far less hospitable (Heinz and Barbaza 1998), and treeless (de Beaulieu et al. 1994).

Pleistocene chronology and nomenclature

For the nomenclature of Pleistocene warm and cold phases we follow the Marine Isotope Stages (MIS) system (Martinson et al. 1987; Lisiecki and Raymo 2005) (Appendix 1: Fig. A1). The main Würm glacier advance (the Last Glacial Maximum, LGM) corresponds to MIS2, the main Riss advance to MIS6. We avoid the terms Mindel and Günz, because there is no consensus on their correlation with MIS stages (cf. Head and Gibbard 2005; Häuselmann et al. 2007). Both probably cover more than one climatic cycle (Kukla 2005). Nomenclature of mountain stocks is according to Marazzi (2005).

Results

Haplotype networks

The main network, based on 1,474 bp of COI (Fig. 2A) contains fifty-three haplotypes, determined by 51 variable sites (two variants per site), accounting for 3.4% of the sequence. Thirty-two sites were parsimony informative, 13 of which were located on the 5'P fragment and 19 on the 3'P

fragment. Twenty-nine substitutions (57%) were transitions. Fifty-one haplotypes (98%) were private to a single subspecies, forty-six (88%) to a single population. Haplotypes are numbered 1 through 53 in Fig. 2A. They appeared grouped in five clusters, designated A through E. Additional haplotypes in the mined sequences of the 3'P fragment are numbered 54 through 59, (inserts in Fig. 2A). The maximum distance (outgroup excluded) was 9 mutational steps (0.61% pairwise sequence difference). The outgroup *Erebia ligea* (haplotypes 60 and 61) was connected by 20 mutational steps, a pairwise sequence difference of 1.35%. The only loop, regarding haplotype 37, was resolved by breaking the connection to cluster C according to the criteria by Crandall and Templeton (1993). The 5'P fragment (Fig. 2B) was hardly discriminating. In the network based on bp 305–1,475 (Fig. 2C), the Sudeten and Carpathian samples retrieved from GenBank appear all in cluster E.

The relationship between subspecies, clusters and haplotypes is shown in Table 2. The clusters in the network turned out largely congruent with morphologic subspecies. Cluster A (Fig. 2A) includes all haplotypes of the western extra-Alpine populations: the Jurassian ssp. *tramelana*, the Pyrenean ssp. *pyraenaeicola* and the Cantabrian ssp. *cantabricola*.

Subspecies *adyte* makes up cluster B, but several *adyte* specimens are placed in cluster E.

Cluster C contains all individuals of ssp. *pseudoadyte*. It has the highest genetic diversity of all subspecies with 11 haplotypes found in two localities.

Cluster D contains only haplotypes of ssp. *kunzi*, but some *kunzi* are assigned to cluster E. Western and eastern *kunzi* are not separated. The Monte Cavallo population (sample 11) builds a separate sub cluster D1, four mutational steps apart from D2 (Fig. 2A), but morphologically indistinguishable from it.

Cluster E is private to four subspecies (*euryale*, *syrmia*, *isarica* and *ocellaris*), and contains individuals of two subspecies that have their own private clusters (*adyte* and *kunzi*). In the first group there is a clear structuring: ssp. *syrmia* is found around the central axis of the network, from which four subclusters, E1 through E4, branch off (Fig. 2A, C). The sub-clusters E2 and E3 are private to ssp. *isarica*, E4 to ssp. *euryale*. Ssp. *ocellaris* occupies E1, but is also represented in E3, together with *isarica*, with which it has a documented hybrid zone (Cupedo 2010). The proportion of individuals with the shared haplotype increases with decreasing distance to the hybrid zone (Table 2). The genetic dichotomy in ssp. *isarica* (E2 and E3) coincides with a geographic-morphologic sub-division. E3 contains the western and northern Alpine populations, E2 the eastern Alpine populations. These groups differ in wing shape and wing pattern (pers. obs. FC).

As to the subspecies with a different private cluster, we suspect that their cluster E haplotypes are due to introgression or incomplete lineage sorting. In sample 4 there is 100% mismatch of phenotype and haplotype. Phenotypically it is an *adyte* enclave in *isarica* territory (Cupedo 2010). The haplotype matches the surrounding *isarica* populations. This fits with previous studies in which gene-flow between these subspecies was not reflected in morphology (Geiger and Rezbanyai 1982). In ssp. *kunzi*, on the other hand, the cluster E individuals were found in the most peripheral samples (7 and 10, Fig. 1), far from the boundary with *ocellaris*, with which they share haplotype 51 (Fig. 2A). This argues against a recent introgression. The fact that both western and eastern *kunzi* are involved, suggests an introgression event predating their separation.

Phylogenetic trees and timing of diversification

Bayesian Inference (BI) and Maximum Likelihood (ML) analyses based on the 1,474 bp alignment (Fig. 3) show similar topologies for the haplogroups A through D, but the position and relationships of haplogroup E are not well supported in any of the trees. The clade comprising ssp. *adyte* and the

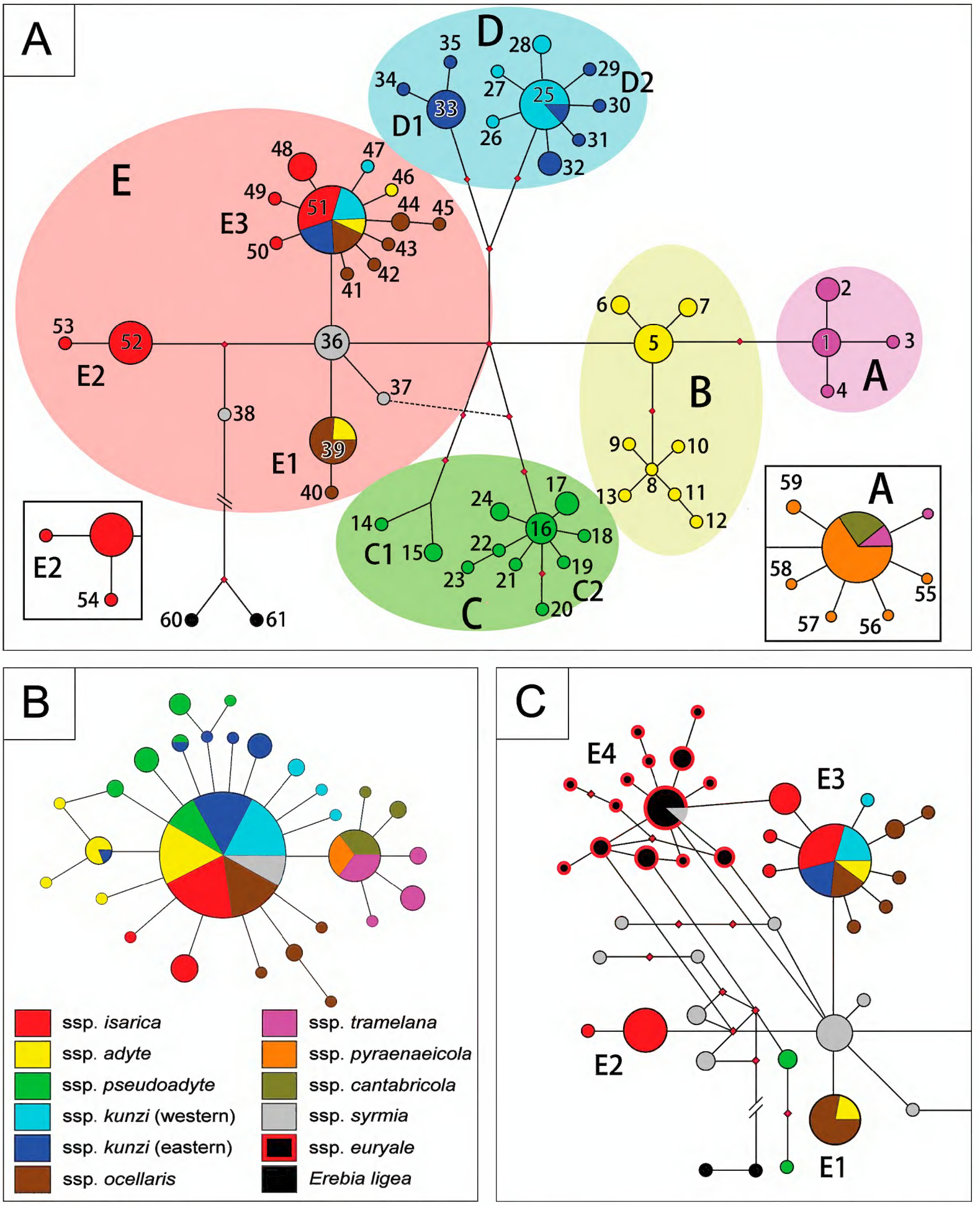

Figure 2. Median–joining haplotype networks, based on different COI segments. Circle diameters are proportional to the frequency of the haplotype. Red diamonds represent hypothetical haplotypes. **A.** Based on 168 3'P+5'P sequences of *Erebia euryale*. Inserts: cluster A and sub cluster E2, based on the 3'P section of the same 168 sequences, together with 77 sequences retrieved from GenBank (Vila et al. 2011). Haplotypes are numbered 1 through 61; **B.** Based on the 5'P section of the same 168 sequences plus 23 sequences retrieved from GenBank (Dincă et al. 2010); **C.** Based on bp. 305–1,475 of the same 168 sequences plus 36 sequences mined from GenBank (Paučulová et al. 2017)**.** In Fig. 2C only the part corresponding to cluster E in Fig. 2A is depicted. Colour legends in frame B apply to Figures 2 to 4.

Table 2. The relationship between subspecies and haplotype, based on the sequences underlying Fig. 2A (sample 1–22 in table 1). **Subsp** – subspecies, syr – syrmia, tram – tramelana, cant – cantabricola, pyr – pyraenaeicola, **Clus** – cluster, **No** – sample number, corresponding to Table 1, **Hpl** – haplotype number, corresponding to Fig. 2A. Field numbers indicate the number of individuals. In bold and italics: discrepancy of phenotype and haplotype.

	Subsp	*adyte*				*pseudoad.*		*kunzi*					*ocellaris*			*isarica*				*syr.*	*tram.*	*cant.*	*pyr.*
Clus	**No**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
	Hpl																						
	1																				5	30	10
	2																				4		
	3																				1		
	4																				1		
A	55																					1	
	56																					1	
	57																					1	
	58																					1	
	59																					2	
	5	3	6	10																			
	6	2																					
	7		2																				
	8	1																					
B	9	1																					
	10	1																					
	11	1																					
	12	1																					
	13	1																					
	14					1																	
	15					2																	
	16					1	5																
	17					4																	
	18					1																	
C	19					1																	
	20						1																
	21						1																
	22						1																
	23						1																
	24						2																
	25							3	10	2													
	26								1														
	27								1														
	28							2															
	29									1													
D	30									1													
	31										1												
	32										4												
	33											9											
	34											1											
	35											1											
	36																			8			
	37																			1			
	38																			1			
	39			***3***									2	8									
	40												1										
	41													1									
	42												1										
	43												1										
	44												2										
E	45												1										
	46			***1***																			
	48															5							
	49							***1***															
	49																1						
	50																1						
	51			***2***				***6***			***6***		4	1	5		10						
	52																	5	11				
	53																		1				
	54																	1					

western extra Alpine subspecies (the network clusters A+B), is well-supported in both trees. The western extra Alpine subspecies are jointly monophyletic with good support, a monophyletic ssp. *adyte* is not supported. There is medium support for a monophyletic ssp. *pseudoadyte*, private cluster C in the network. The clades D1 and D2 are well supported in both trees, the monophyly of D1+D2, however, is not well supported in the BI analysis and only moderately supported by ML. The monophyly of individual clades E1 and E2 is also well supported in both analyses, E3 somewhat less so in BI, but their positions in the trees are not well resolved. Overall, the BI with molecular dating suggests that the diversification into the present subspecies took place between ~0.75 and ~2 My ago.

Discussion

Three of our specific questions have implicitly been resolved.

(i) Both in the haplotype network (Fig. 2A), in the BI tree and in the ML tree (Fig. 3) the subspecies *pseudoadyte* and *kunzi* appear as distinct lineages, different from the morphologically similar ssp. *adyte* and *ocellaris*.

(ii) Western *kunzi*, morphologically intermediate between *pseudoadyte* and nomotypical (eastern) *kunzi*, is genetically inseparable from the eastern form (Fig. 2A) and has correctly been ascribed to ssp. *kunzi*.

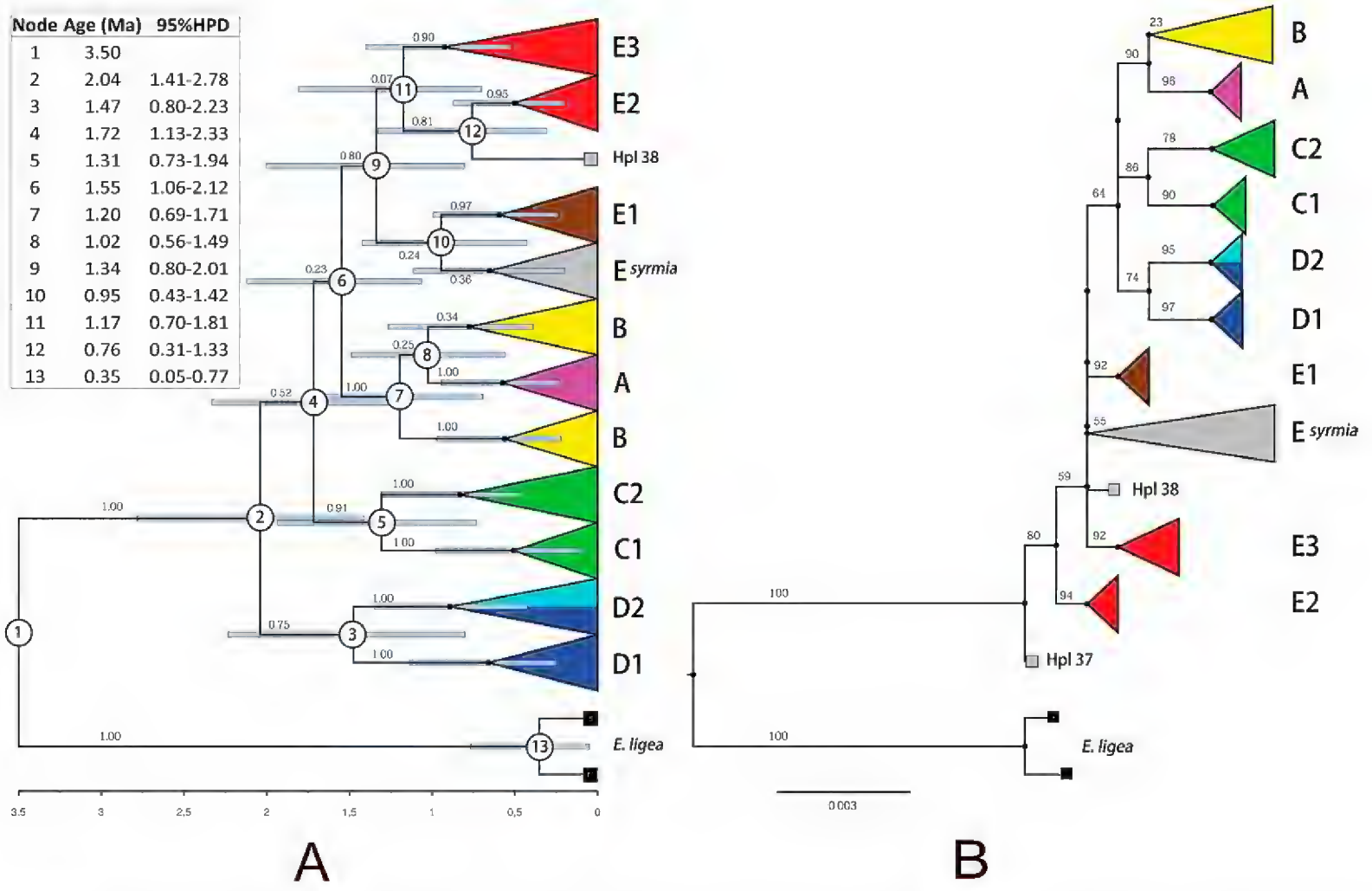

Figure 3. Phylogenetic trees based on the haplotypes in Fig. 2A, with clusters and sub-clusters collapsed into triangles. Colour codes as in Fig. 2, cluster labels as in Fig. 2A. **A.** Bayesian Inference tree. Support values on the branches indicate the Bayesian posterior probabilities. Blue node bars indicate the 95% posterior density for the Bayesian age estimates. Insert: estimates of the divergence time from the RevBayes approach, based on a calibrated split at 3.5 My BP between *E. euryale* and *E. ligea* (Peña et al. 2015). The time scale is in My ago. **B.** Maximum Likelihood tree. Support values on the branches indicate bootstrap support (5,000 replicates).

(iii) Western ssp. *isarica* and ssp. *ocellaris* share a single haplotype (Table 2). Eight haplotypes derived from the shared one, by one or even by two mutations, are private. In addition, ssp. *ocellaris* has two haplotypes in the private sub cluster E1. With one out of seven (*isarica*) or eight (*ocellaris*) haplotypes shared (Table 2), we consider *isarica* and *ocellaris* distinct genetic lineages. The conclusion that they are genetically inseparable (Schmitt and Haubrich 2008) was based on the Kalkstein population, which is inseparable from *isarica* by its mtDNA as well (Table 2).

Linking lineages to Würm glacial refugia

The actual distribution pattern results from Holocene range extensions, originating in the Würm glacial refugia. Overlaying the potential Würm refugia with the current subspecies distributions shows a clear matching pattern.

Ssp. *adyte* occupies the entire southwestern Alps (Fig. 1). Its distribution area stretches between two known biogeographical borders (Cupedo and Doorenweerd 2020): the Isère valley in the west and the Valtellina (the Adda valley) in the east, suggesting survival in refugium (i) and in the western compartments of refugium (iii).

The territory of ssp. *pseudoadyte* is sharply delimited by the valleys of Adda and Adige (Fig. 4), matching a single compartment of refugium (iii). Its postglacial northward expansion was halted at the Adda and Adige valleys, which currently delimit its distribution.

As to ssp. *kunzi*, all former peripheric nunataks between Val d'Adige and Valcellina, belonging to refugium (iii), harbour relict populations of this ssp. (Cupedo 2010; Bonato et al. 2014): the Monti Lessini, Monte Grappa, Col Visentin and Monte Cavallo (Fig. 4). Sub-cluster D1 to date is confined to its refugial massif, the Monte Cavallo, and is separated from D2 by the Piave valley

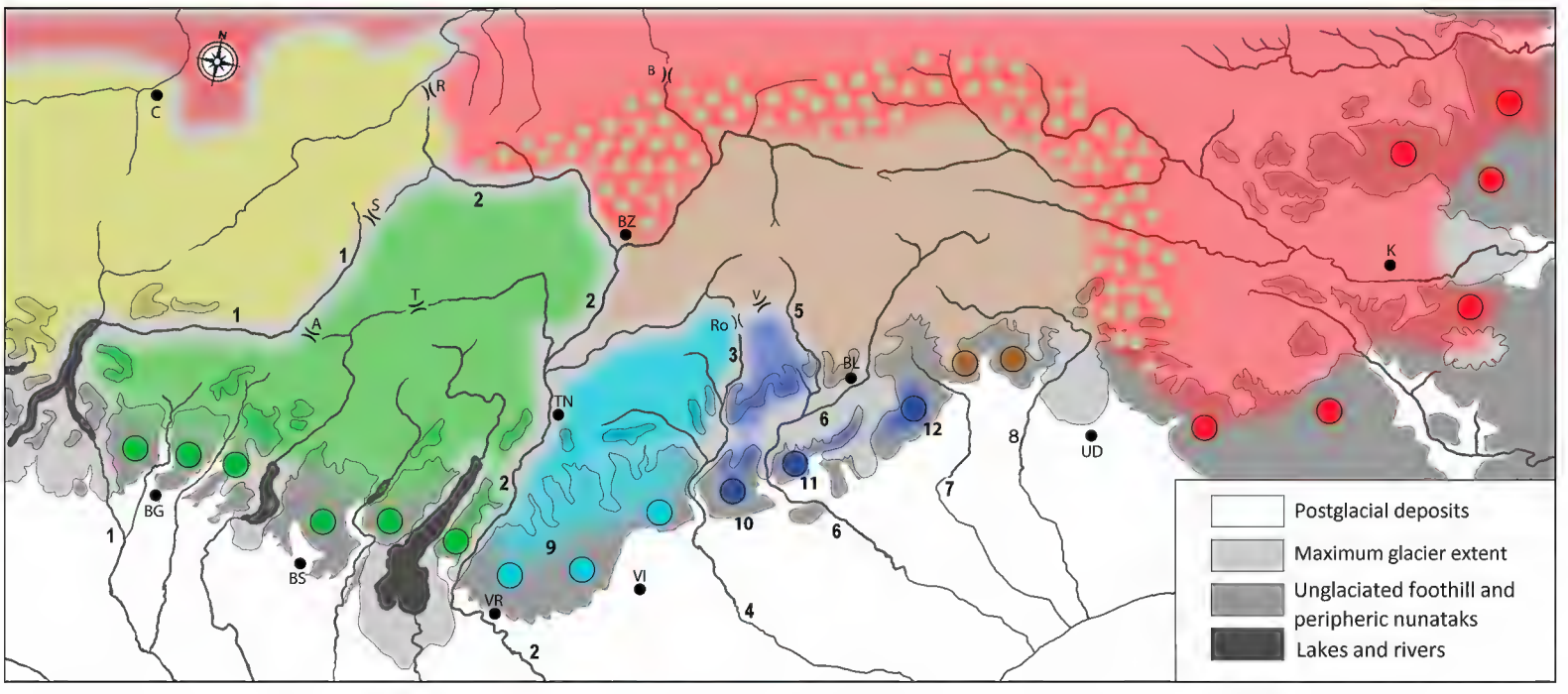

Figure 4. The southern Alps east of Lake Como, showing the relationship between the current distribution (coloured areas) and hypothesized refugial areas (coloured dots) of the subspecies of *E. euryale* during the maximum Würm glaciation (MIS2). Dotted area – transitional populations *isarica/ocellaris*. Colour legends as in Fig. 2.

(Fig. 4). Only sub-cluster D2, living across the Piave valley, could invade the southern Dolomites, where it failed to cross the deeply incised Cordevole valley (Fig. 4). Eastern and western D2, only differing in wing pattern, are separated by the Cismon-Brenta valley.

The ssp. *ocellaris* territory is considerably larger than that of ssp *kunzi*, but it includes only a short section of the southern pre-Alpine chain (Figs 1, 4). Its refugium along the Alpine border most probably stretched from the Valcellina to the Tagliamento valley, matching the eastern part of refugium (iii).

Ssp. *isarica* has by far the largest distribution of the Alpine subspecies (Fig. 1). We suppose that the two COI sub clusters represent populations originating from different glacial refugia. The current territory of cluster E2 comprises the vast refugial area (ii) on the eastern edge of the Alps (Fig. 4), whereas cluster E3 suggests refugia along the northern outskirt of the Alps (refugium iv), the exact locations of which remain unknown.

Reconstruction of pre-Würm phylogeography

The five-fold cluster structure of the network is superimposed by a geographic tripartition. We distinguish (i) a southern Alpine group, consisting of the clusters B, C and D, (ii) a circum–Pannonian group, coinciding with cluster E, in which Carpathian populations are combined with northern and eastern Alpine ones and (iii) a western extra Alpine group, equalling cluster A.

Ad (i). In the southern Alpine group the subspecies *adyte*, *pseudoadyte* and *kunzi* occupy adjacent territories. They share a sharply defined, coherent territory and a considerable genetic variation. The pairwise sequence difference within the clusters ranges from 0.68% to 1.15%. This suggests a long-term isolation within the actual distributional borders. Most probably the increasing incision of the dividing valleys, by repeated glacial erosion, resulted in a permanent vicariance, enabling their differentiation.

Ad (ii). In the circum–Pannonian group, the core area of cluster E in the haplotype network is occupied by the Southern and Eastern Carpathian ssp. *syrmia* (Figs 1A, 2A). Sub-clusters radiate from it into the Western Carpathians (ssp. *euryale*, Fig. 2C) and into the Alps (ssp. *isarica* and ssp. *ocellaris*, Fig. 2A).

This division into a southern Alpine group and a circum–Pannonian group suggests an ancient Alpine–Carpathian disjunction. This is in agreement with earlier allozyme data: Schmitt and Haubrich (2008) found the split between the Carpathian and Alpine lineages (in their study: ssp. *syrmia* and ssp. *adyte*) the most ancient disjunction in the studied *E. euryale* populations. Such an Alpine–Carpathian disjunction is typically found in species of humid grasslands of the montane-subalpine levels (Varga and Schmitt 2008), and is associated with a Pannonian refugium (refugium v). The common ancestor could, in theory, have been resident either in the Alps or in the Carpathians. The close genetic affinity between the outgroup *E. ligea* and the Carpathian samples in the network (Fig. 2A) suggests a Carpathian origin, but the phylogenetic tree lacks the statistical support to corroborate this scenario.

Subsequently, i.e., during subsequent cold periods, the southern Alpine group differentiated into its three lineages (*adyte*, *pseudoadyte* and *kunzi*), and the circum-Pannonian group split up into a Carpathian and an eastern Alpine lineage. This required a second, more recent glacial retreat in the Pannonian plain, again followed by interglacial invasion of both the Alps and the Carpathians. Finally, the Sudeten and Carpathian populations differentiated into what is now known as ssp. *euryale* and ssp. *syrmia*, while the Alpine populations differentiated into today's ssp. *ocellaris*, eastern *isarica* and western *isarica*. We presume that this occurred during MIS2, when the refugia

of the Alpine populations were localised in the Alpine periphery rather than in the Pannonian plain (Fig. 4), because the Alpine glacier extension was far less than during the foregoing glaciations (Penck and Brückner 1909; Castiglioni 2004; van Husen 2004).

Ad (iii) The western extra-Alpine group. Both the network and the ML tree suggest that it derived from cluster B (Figs 2A, 3B). Its star-like configuration, with many low frequency haplotypes derived from a most common one, is typical of a relatively recent demographic expansion after a genetic bottleneck (Rogers and Harpending 1992; Avise 2000; Hwang and Cho 2018). This bottleneck probably was associated with its separation from the Alpine cluster B (Fig. 2A). Because an Alpine–Pyrenean disjunction required sub alpine conditions in the in-between Rhone basin, it must have been associated with a glacial period. Because of the identical genetic content of the Pyrenean and Jurassian populations, we exclude two independent colonisation events. We assume that either the Pyrenees and the Jura were colonised simultaneously, or the Jura was colonised secondarily from the Pyrenees.

However, nuclear data suggest a somewhat different scenario. Schmitt and Haubrich (2008) found the Pyrenean population genetically closer to *isarica* than to *adyte*. Although their data refer only to eastern *isarica*, this suggests a connection to the northern, rather than to the southern Alps. This mismatch between mitochondrial and nuclear data calls for further study on the origin of the western extra-Alpine group of *E. euryale*. In general, mitochondrial and nuclear DNA need not have responded similarly to past biogeographic events, and their patterns of variation provide complementary data on the phylogeographic history (Hinojosa et al. 2019). We expect additional genomic data to enhance the insight in the origin of the western extra-Alpine populations.

Morphology

Some morphologic taxonomic characters have been shown phylogenetically relevant, provided they are combined with molecular data. (Wahlberg and Nylin 2005; Simonsen et al. 2006; Pisani et al. 2007; Stephanović et al. 2016). In our study, the three morphologic subspecies that make up the southern Alpine group (*adyte*, *pseudoadyte* and *kunzi*) appeared congruent with well separated haplotype clusters (Fig. 2A). These three subspecies (together with the Apennine ssp. *brutiorum* which is not included in this study), share a distinctive character: they have white pupiled apical ocelli on the forewing upper side. All remaining subspecies (including the boreal/tundral populations, not studied either), have uniform black ocelli. The pupiled ocelli appear to be a synapomorphy of the southern Alpine group. This supports the conclusion that the separation of the southern Alpine group was a basal split in the history of *Erebia euryale*. It further suggests that the Apennine populations are derived from the southern Alpine group, and that the boreal/tundral populations are derived from the circum-Pannonian group. Both hypotheses are consistent with geography. Moreover, it supports the alternative, allozyme based scenario for the origin of the western extra-Alpine group, in which the apical ocelli are black.

Overall timeline of diversification

The Bayesian estimate of divergence times, using an external calibration, dates the differentiation of the crown group roughly 0.75 My to 2 My ago, i.e., in the early Pleistocene (Appendix 1: Fig. A1). However, the correlation of lineage splits to glacial conditions provides an alternative calibration for the age of splitting events (Schmitt et al. 2016; Hinojosa et al. 2018). For the relatively young Pyrenean disjunction we assume at least a MIS6 age, because there is some evidence for more recent (i.e., MIS2) differentiations within this cluster: (i) genetic differences between populations at both sides of the Cantabrian watershed are a possible indication of two Würm glacial refugia in the

Cantabrian region (Vila et al. 2011). (ii) Likewise, the morphological differentiation of the Pyrenean populations into ssp. *antevortes* in the north-western Pyrenees (not investigated here) and ssp. *pyraenaeicola* in its southern and eastern parts, must be younger than the Alpine–Pyrenean disjunction.

Because the differentiation of the clusters B, C and D in their southern Alpine refugia predates the Alpine–Pyrenean disjunction (Fig. 3), it is at the latest of MIS10 age. The initial Alpine–Carpathian disjunction, necessarily associated with a severe cold phase, thus most probably is not younger than MIS12. We emphasise that the ages of disjunctions reconstructed this way are always minimal ones and splitting events might prove considerably older.

The dependency of major cold phases, however, sets a maximum age too. It has been shown that tributary glaciers in the eastern Alps did not reach the main valleys during minor cold periods like MIS4 (van Andel and Tzedakis 1996; van Husen 2004), therefore subalpine organisms were not dispelled into refugial isolation. This was only the case during the more severe cold phases. The very first of those major ice ages was MIS22, ca 870–880 ka ago (Ehlers and Gibbard 2007) (Appendix 1: Fig. A1). All foregoing cold phases were in the order of MIS4 or less. Consequently, it is unlikely that the first Alpine–Carpathian disjunction took place more than 1 My ago. This conflicts with our Bayesian dating (Fig. 3A), secondary calibrated with Peña et al. (2015). It is in line, though, with recent molecular studies on the *E. tyndarus* group (Schmitt et al. 2016) and the *E. epiphron–orientalis* group (Hinojosa et al. 2018). Both calculated divergence times, in the Pliocene–Pleistocene period, that are considerably younger than those inferred from secondary calibrations based on the external calibration from Peña et al. (2015).

Conclusions

In *Erebia euryale* the 3'P section of the COI gene is far more discriminative than the 5'P section. It revealed the presence of five intraspecific clades, three of which remained hidden using the barcode section alone. The clades are congruent with morphologic subspecies. One of these, ssp. *isarica*, seems even composed of two genetically different lineages. That makes six lineages in the Alps, four of which were not recognised earlier.

All subspecies addressed here, i.e. all but the boreal component and the Apenninian populations, are derived from a common ancestor, supposedly resident in the Carpathians. This ancestral population split up into two main clades, an Alpine one and a Carpathian one, probably no more than 1 My ago. This dichotomy is consistent with a glacial refugium in the Pannonian plain. During mid-Pleistocene, partitioning of the southern Alps by glacial valleys led to the differentiation of three lineages: *adyte*, *pseudoadyte* and *kunzi*. The Carpathian branch colonised, after a second refugial retreat in the Pannonian plain, both the Carpathians and the eastern Alps. In a subsequent cold period the Alpine populations differentiated into western *isarica*, eastern *isarica* and *ocellaris*, whereas the Carpathian populations differentiated into *euryale* and *syrmia*. In the western Alps, a Pyrenean–Jurassian clade was separated from an Alpine clade, at the latest during MIS6. Our COI data support a split from *adyte*, but allozyme data endorse a western *isarica* rooting. Postglacially, the southern Carpathian ssp. *syrmia* spread into the Balkans, and the Alpine ssp. *isarica* and *ocellaris* built a broad secondary contact zone. There is, however, no doubt that additional genomic data in future work will enhance the resolution of the relationships and of the timing of splitting events. The use of nuclear genomic data would also address the possibility that the divergences we detected in COI have been influenced by *Wolbachia* infections, which have been detected in *E. Euryale* too (Ritter et al. 2013; Lucek et al. 2021).

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Appendix 1

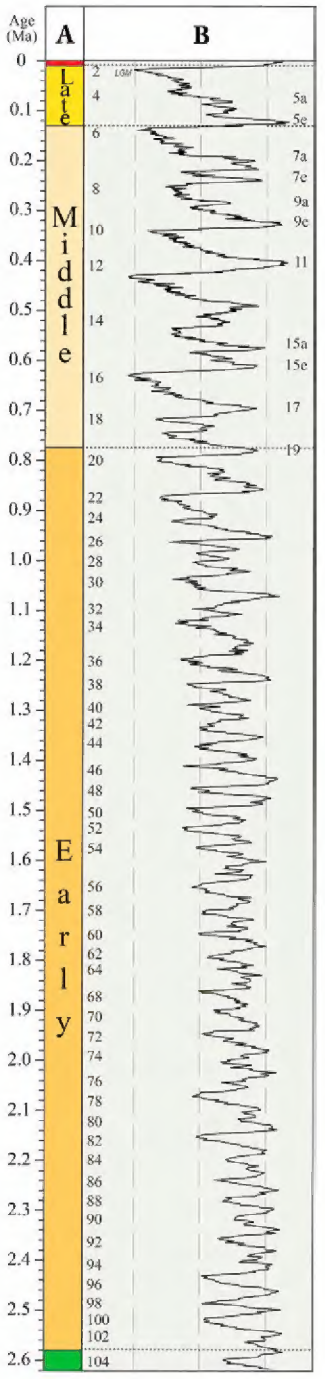

Figure A1. Global temperature curve of the Quaternary period, based on the $^{16}O/^{18}O$ ratio in benthic Foraminifera (Martinson et al. 1987; Lisiecki and Raymo 2005). **A.** Epoch; **Red** – Holocene, **yellow/orange** – Pleistocene, **green** – Pliocene. **B.** Temperature curve. Flanking numbers indicate MIS stages: even numbers (left)=cold stages, odd numbers (right)=warm stages. From Cohen and Gibbard (2019), modified.

Supplementary material 1

Table S1

Authors: Frans Cupedo, Camiel Doorenweerd

Data type: Table (excel file).

Explanation note: Table S1. Partial COI sequences, mined from GenBank. Morphological subspecies, accession numbers, geographical locations, and number of individuals per sequence.

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